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*Research Note*

# Development of *Onchocerca cervicalis* to the Third Larval Stage in *Simulium pictipes*

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ABSTRACT: The objective of this study was to assess the ability of a temperate black fly, *Simulium pictipes,* to support larval development by *Onchocerca cervicalis,* a filarial parasite of horses normally associated with ceratopogonid vectors. A proportion of both freshly isolated and cryopreserved microfilariae (MF) inoculated intrathoracically into *S. pictipes* completed development to the third larval stage (L3) in the thoracic and cephalic musculature. The first and second molts occurred as early as days 6 and 7 of infection, respectively. Relative to the course of *O. cervicalis* infection in a natural vector, *Culicoides nubeculosus,* the frequency of development in *S. pictipes* was low. Black flies inoculated with 50 freshly isolated MF ultimately yielded a mean of 3.3 L3 per fly, representing 6.5% of the original inoculum. In contrast, 2.1% of the cryopreserved MF were recovered as L3. Migration of L3 from the thoracic musculature to the heads and mouthparts of infected flies could not be ascertained. The mean length of L3 recovered from *S. pictipes* was 579 μm, approximately 35% shorter than L3 derived from the natural vector. These results demonstrate that *S. pictipes* will support development of a proportion of inoculated *O. cervicalis* MF, making it a potential source of these parasite stages albeit an inefficient one. The relevance of this finding to the development of laboratory models for the study of onchocerciasis is discussed.

KEY WORDS: *Onchocerca cervicalis, Simulium pictipes,* black fly, microfilaria, third-stage larva, surrogate vector.

Efficient production of *Onchocerca volvulus* Leuckart, 1893 third stage larvae (L3) for laboratory research has constituted a major obstacle in the development of vaccines, macrofilaricides, and chemoprophylactics against human onchocerciasis. Onchocercids of livestock such as *O. lienalis* Stiles, 1892; *O. ochengi* Bwangamoi, 1969; and *O. gibsoni* Cleland and Johnston, 1910 in cattle, and *O. cervicalis* Railliet and Henry, 1910 in horses, have been proposed as models for basic research in this area (Copeman, 1979; Jones and Collins, 1979; Lok and Abraham, 1992; Trees, 1992). Temperate black flies such as *Simulium pictipes* Hagen, 1880 and *S. ornatum* Meigen, 1818 make highly efficient laboratory vectors for *O. lienalis* when inoculated intrathoracically with microfilariae (MF) (Lok et al., 1983; Bianco et al., 1989). It is noteworthy that these temperate black flies also support development of the allopatric, black-fly-associated filariae, *O. volvulus* (Ham and Bianco, 1983; Lok, et al., 1983) and *O. ochengi* (McCall and Trees, 1989), to the L3. *Culicoides* spp., the natural vectors of *O. cervicalis,* are highly susceptible to experimental infection with isolated MF of *O. cervicalis* via the oral route (Mellor, 1975; Collins and Jones, 1978), but to date no system of mass production of *O. cervicalis* L3 utilizing these flies has been demonstrated. The objective of this study was to determine whether the black fly *S. pictipes* could serve as an alternate source of *O. cervicalis* L3 for laboratory study.

Late larval instars of *S. pictipes* were collected from a bedrock cascade in Bushkill Creek near Resica Falls, Pennsylvania, U.S.A. These larvae were reared to adults in a closed-circulation rearing system as described by Brenner and Cupp (1980). Three initial infection trials were carried out with a batch of *O. cervicalis* MF collected from infected horses at slaughter, cryopreserved, and later thawed according to the technique of Ham et al. (1981). For a fourth infection trial, a fresh umbilical skin from an infected horse was shipped on wet ice via overnight courier from its collection site in South Carolina to the University of Pennsylvania, where MF were extracted and used immediately.

Freshly isolated or cryopreserved MF were suspended in Ham's Medium F12, containing

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**Table 1. Findings from dissection of *Simulium pictipes* experimentally infected with 50 *Onchocerca cervicalis* microfilariae and incubated at 27°C.**

| Day of infection | Treatment of MF* | No. of flies dissected | % flies infected | No. of larvae recovered | Breakdown by stage | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | MF | L1 | L2 | L3 |
| 1 | Cryopreserved | 6 | 66.7 | 47 | 47 | — | — | — |
| 2 | Cryopreserved | 8 | 12.5 | 8 | 8 | — | — | — |
| 3 | Cryopreserved | 15 | 40.0 | 12 | 2 | 10 | — | — |
| 4 | Cryopreserved | 14 | 71.4 | 27 | — | 27 | — | — |
| 5 | Cryopreserved | 4 | 100 | 18 | — | 18 | — | — |
| 6 | Cryopreserved | 3 | 66.7 | 12 | — | 3 | 9 | — |
| | Fresh | 5 | 100 | 46 | — | 8 | 38 | — |
| 7 | Cryopreserved | 21 | 66.7 | 34 | — | 2 | 30 | 2 |
| | Fresh | 3 | 100 | 14 | — | 1 | 13 | 1 |
| 8 | Cryopreserved | 16 | 62.5 | 22 | — | 1 | 19 | 2 |
| | Fresh | 3 | 100 | 33 | — | — | 12 | 21 |
| 9 | Cryopreserved | 8 | 50.0 | 8 | — | — | 2 | 6 |
| | Fresh | 3 | 100 | 33 | — | 1 | 16 | 16 |
| 10 | Cryopreserved | 10 | 50.0 | 11 | — | — | 5 | 6 |
| | Fresh | 5 | 100 | 42 | — | — | 15 | 27 |
| 11 | Cryopreserved | 6 | 50.0 | 6 | — | — | 2 | 4 |
| 12 | Fresh | 3 | 100 | 9 | — | — | 4 | 5 |

* Data on cryopreserved MF are pooled from 3 cohorts of flies infected with parasites from the same cryopreservation lot. Data on fresh MF are from a single infection cohort.

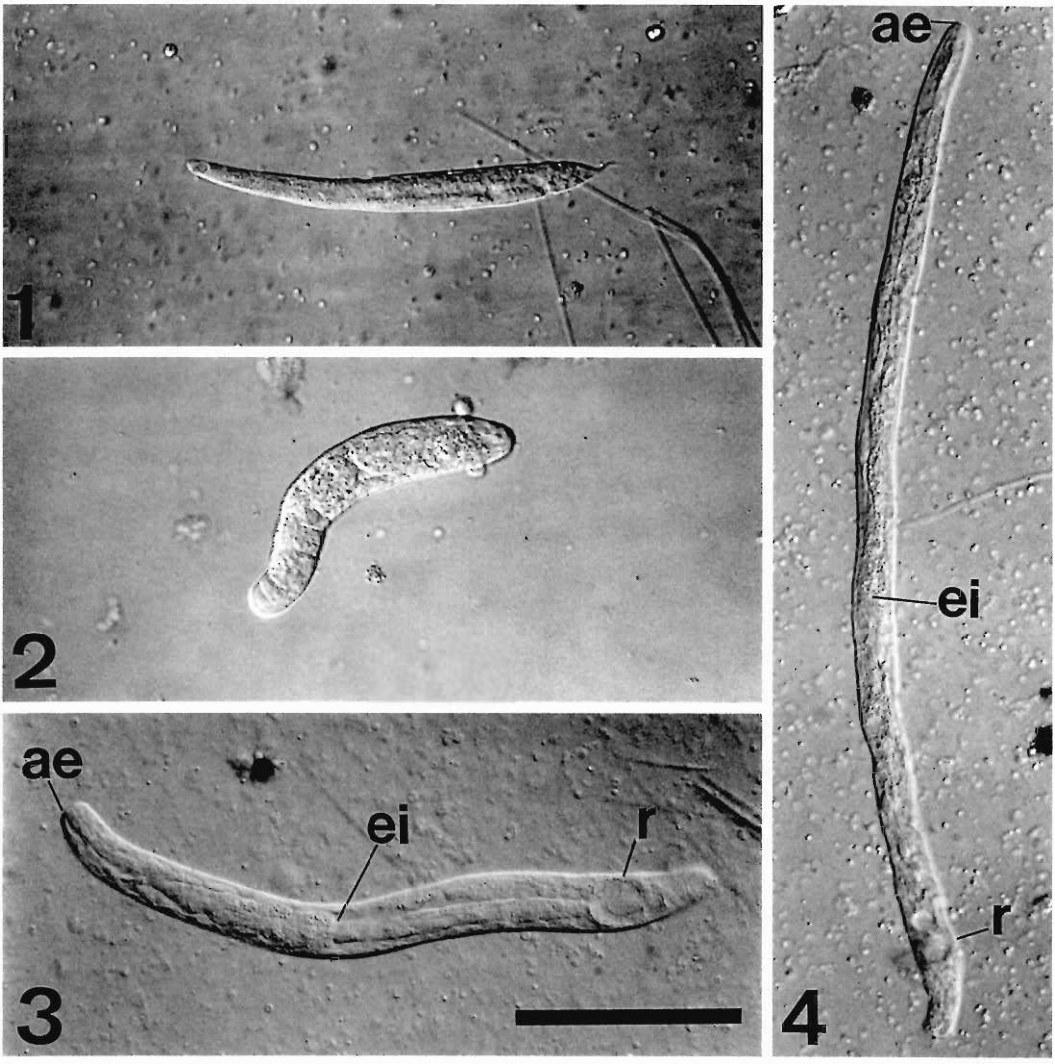
20% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B, and injected intrathoracically into female *S. pictipes* at a dose of 50 MF per fly using finely drawn glass micropipets as described by Lok et al. (1983) and Bianco et al. (1989). Flies were injected 24–48 hr after eclosion. Inoculated flies were incubated in humidified cages at 27°C and fed on a 30% sucrose solution via a filter paper wick.

Each day, a sample of live flies was dissected and examined for developing larvae. In the trial involving freshly isolated MF, dissections were postponed until day 6 of infection in order to conserve inoculated flies and thus obtain a more accurate assessment of the system's potential to generate L3. Numbers of larvae in the head, thorax, and abdomen of each fly were recorded, and the parasites were measured and photographed under Nomarski differential interference contrast microscopy. They were assessed as to stage of development with the aid of morphological descriptions given by Mellor (1975) and Bain and Petit (1978). Dead flies were removed daily from the cohorts, counted, and discarded.

Quantitative findings from dissection of *S. pictipes* inoculated with *O. cervicalis* appear in Table 1. At 27°C the majority of larvae had matured to the "sausage form" first stage (L1; Figs. 1, 2) by day 3 of infection. In most cases, the molt to the second stage (L2; Fig. 3) commenced on day 6 and the molt to the L3 (Fig. 4) commenced on day 7. Larval development was asynchronous, with L3 appearing as early as day 7 and L2 persisting as late as day 12. The total number of larvae, regardless of stage, recovered after inoculation of cryopreserved MF represents a developmental rate of 5.7%. The total number of L3 recovered represents 2.1% of the original inoculum. Much higher infection rates and parasite burdens were seen in flies infected with freshly isolated MF. The numbers of developing larvae and L3 recovered from flies inoculated with fresh MF (Table 1) represent 17.5% and 6.5% of the original inoculum, respectively.

There were frequent instances of abnormal development. Midbody constrictions and vacuolation of the posterior intestine were occasional findings, and examples of incomplete ecdysis were also seen.

The anatomical distribution of larval *O. cervicalis* recovered from *S. pictipes* is given in Table 2. At the time of injection, MF were dispersed throughout the heads, thoraces, and abdomens of the black flies, and the distribution of the MF among these segments remained random for approximately 48 hr. Although the majority of preinfective larvae were recovered from the thoraces of infected flies, low percentages of L1 and L2 as well as L3 were found in the heads. In




**Figures 1–4. Developing larvae of *Onchocerca cervicalis* from the thoracic musculature of *Simulium pictipes* after intrathoracic inoculation with cryopreserved microfilariae. 1. Early L1, day 4 of infection. 2. Late L1, day 7 of infection. 3. L2, day 8 of infection. 4. L3, day 9 of infection. Bar = 100 μm. Abbreviations: ei = esophageal/intestinal junction, ae = anterior extremity, r = rectum.**

addition, a small number of L3 were found in the abdomens of flies on day 12 of infection with fresh MF.

High mortality occurred in the inoculated black flies between days 0 and 2 and days 6 and 8 (Fig. 5). Survival declined to 50% by day 5 of infection. By days 8 to 10 of infection, when significant numbers of L3 were seen, it was 10% or less.

These results demonstrate that MF of *O. cervicalis* can develop to the L3 when injected intrathoracically into *S. pictipes*. Infectivity of L3 derived in this manner remains to be ascertained. The maximum efficiency of conversion of *O. cervicalis* MF to L3 in *S. pictipes* was 6.5%. This developmental rate was low considering that approximately 20% of *O. lienalis* MF develop to the L3 in this vector (Lok et al. 1983). On the other hand, developmental rates in the *O. cervicalis/S. pictipes* system are high compared with those seen in another surrogate vector, *Aedes aegypti,* in which only 1.6% of fresh, injected *O. cervicalis* MF complete development to the L3 (Lok et al. 1980). Comparable data on *O. cervicalis* in *Culicoides* sp. are not available. The

**Table 2. Anatomical distribution of *Onchocerca cervicalis* larval stages in experimentally infected *Simulium pictipes*.**

| Larval stage | No. of parasites observed* | % of recovered parasites per body segment | | |
|---|---|---|---|---|
| | | Head | Thorax | Abdomen |
| MF | 57 | 19.3 | 19.3 | 61.4 |
| L1 | 73 | 4.1† | 95.9 | — |
| L2 | 174 | 9.2† | 90.8 | — |
| L3 | 92 | 6.5† | 90.2 | 3.3‡ |

* Pooled data from all infection trials; fresh and cryopreserved MF included.

† Numbers of L1, L2, and L3 recovered from heads not significantly different ($\chi^2 = 1.96$; $P = 0.38$).

‡ L3 recovered day 12 of infection with fresh MF.

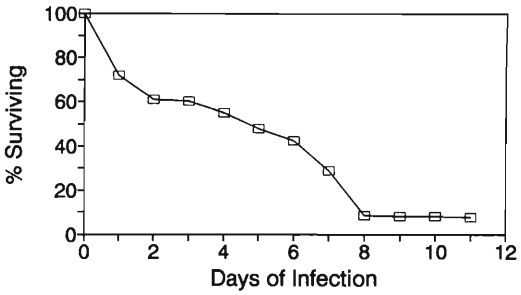



**Figure 5. Survival of *Simulium pictipes* inoculated intrathoracically with microfilariae of *Onchocerca cervicalis*.**

fact that extraction and purification of MF designated as "fresh" and "cryopreserved" were carried out in different laboratories does not permit a rigorous comparison of the infectivity of fresh and cryopreserved *O. cervicalis* MF in the context of this study. Given the wide applicability of the specified technique for cryopreservation (Ham et al., 1981) it seems likely that the observed differences in developmental success were due to batch variation in technique and not to the amenability of *O. cervicalis* MF to cryopreservation per se. Maintenance of the infected skin sample containing fresh MF on wet ice for a period of almost 24 hr during overnight shipment represents a departure from our standard technique for collection of *O. lienalis* MF, where skins are maintained at room temperature and are processed within 6 hours of collection. This technical factor may have resulted in diminished infectivity of fresh MF in the present study.

The mean length (±SD) of the MF used in the present study (223.7 ± 27.6 μm; $N = 6$) was equivalent to published values for *O. cervicalis* (Mellor, 1975). However, at a mean length of 579 ± 80 μm, the L3 derived from *S. pictipes* were shorter than ceratopogonid-derived L3, which range in length from 680–870 μm (Mellor, 1975; Bain and Petit, 1978). The lengths of L3 in the present study more closely approximate the mean length of 558.8 ± 32.2 μm reported for *O. cervicalis* L3 in *Aedes aegypti*. Thus, it appears that stunting of *O. cervicalis* L3 can occur as a result of development in an abnormal vector. Voucher specimens of *O. cervicalis* L3 recovered from *S. pictipes* have been deposited in the U.S. National Parasite Collection, USNPC No. 84909.

Aberrant and retarded development similar to that observed in the present study have been reported for *O. cervicalis* in a natural vector, *Culicoides nubeculosus* (Mellor, 1975) and *Ae. aegypti* (Lok et al., 1980). Melanotic encapsulation, which figured prominently in abortive development by *O. cervicalis* in *Ae. aegypti*, was not observed in *S. pictipes*.

Another common feature of *O. cervicalis* infections in *S. pictipes* was the failure of fully formed, active L3 to migrate to the heads and mouthparts of the flies after 12 days' development at 27°C. The small number of L3 observed in the heads of these flies can be accounted for fully by the numbers of L1 and L2 apparently developing in this body segment. Development of larval filariae in the cephalic musculature has been observed in other vector–filaria systems. Laurence (1985) described the development of larval *Brugia patei* in the pharyngeal musculature of an experimental mosquito vector, *Ae. togoi*. The fact that *C. variipennis* discharge a small number of *O. cervicalis* L2 during bloodfeeding on an artificial membrane (Collins and Jones, 1978) also suggests that preinfective larvae of this parasite may reside in the head of the vector. The finding of a few L3 in the abdominal hemocoels of flies 12 days after inoculation with fresh MF may indicate the beginning of migration by L3 at this time. However, high mortality among infected *S. pictipes* made it unfeasible to harvest significant numbers of parasites after periods of incubation longer than 12 days. Survivorship among black flies in the present study was much lower than in *S. pictipes* inoculated with *O. lienalis* previously (80% at day 12; Lok et al., 1983). This observation may indicate relatively high pathogenicity of *O. cervicalis* in *S. pictipes*. However, in the absence of the appropriate experimental controls, the hypothesis that low black

fly survival in the present study was due to substandard injection or rearing technique must be given equal weight.

The failure of L3 to migrate normally in *S. pictipes,* the stunting of the recovered L3, and the low rate of parasite development support a characterization of this black fly as an inefficient surrogate vector for *O. cervicalis.* Nonetheless, the present findings indicate that, until a practical system of mass production involving ceratopogonids is developed, *S. pictipes,* and perhaps other temperate black flies, may act as an alternative source of *O. cervicalis* L3. These findings also raise the prospect that black flies could serve as a source of L3 of another ceratopogonid-associated onchocercid, *O. gibsoni.*

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